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(57) Abstract

The present invention relates to DNA sequences, expression cassettes and DNA constructs for use in therapy, specifically in gene therapy for the treatment of infectious diseases such as mastitis. Also included are pharmaceutical and veterinary compositions containing the constructs, and cells which have been transformed with the DNA and which are suitable for implantation into a host mammal. The gene therapy of infectious diseases can be effected *in situ* in targeted tissue or systemically.

ANIMAL GENE THERAPY

BACKGROUND OF THE INVENTION

(a) Field of the Invention

5   The invention relates to DNA sequences, expression cassettes and DNA constructs for use in therapy, specifically in gene therapy for the treatment of infectious diseases such as mastitis. Also included are pharmaceutical and veterinary compositions containing

10   the constructs, and cells which have been transformed with the DNA and which are suitable for implantation into a host mammal.

(b) Description of Prior Art

At the highest level, transgenic animals are

15   the principal way to confer transmissible resistance to diseases in animals. Only few years after the first successful gene transfer into mice the new technique was used in farm animals. Several genetic treats have been targeted for the application of transgenesis in

20   domestic animals, but one of those important aspects is the improvement of animal health and disease resistance by gene transfer means. Transient as well as stable genetic improvement leading to disease resistance and treatment achieved by recently developed techniques in

25   molecular biology may contribute considerably to reduce the problem of diseases.

Resistance to infections in animals elicited at various levels. Constitutional and phagocytic mechanisms (innate immunity) serve as a first line of

30   defense. If these are ineffective the infected organism can respond by means of specific (acquired) immunity. Thus, candidates for gene therapy applications include all genes known to modulate non-specific and specific host defense mechanisms, i.e. cytokines, major

35   histocompatibility complex (MHC) proteins, T-cell receptors (TCR) and proteins conferring specific dis-

ease resistance.   Increased protection against patho-
gens can be conferred also by other strategies such as
"intracellular immunization", genetic immunization,
antisense sequences as anti-pathogenic agents and dis-
5   ruption of disease susceptibility genes.

Gene modulating Immune Responses

         Cytokine orchestrate immune responses through
their role as soluble mediators of cell communication.
10   Initially identified to direct viability, prolifera-
tion, differentiation and homing of leukocytes, they
were also found to regulate the production of function
or one another.   In addition, cytokines interact with,
and are produced by cells other than leukocytes, thus
15   providing a means of communication between the immune
system and other tissues and organs.   Cytokines repre-
sent a rapidly growing number of regulatory peptide
factors including growth factors, interleukins,
chemokines, colony-stimulating factors and interferons.
20   Their functions are mediated through binding to cell
surface receptors on their target cells.   Cytokines
have been shown to contribute directly to the develop-
ment of pathology during infectious diseases and
tumorigenesis.   Different cytokines have been reported
25   to both positively and negatively influence host
defense mechanisms.

         Interferon (IFNs) are a well characterized
class of cytokines eliciting antiviral and antiprolif-
erative activity as well as modulating cell growth,
30   differentiation and immune responses.   As well as their
more characterized antiviral activity, IFNs are instru-
mental in counteracting non-viral pathogens mostly
through their effects on macrophage activation.   The
proteins known to be involved in the antiviral and bac-
35   tericidal actions of interferon and their inhibitory
mechanisms are numerous.   The potency of IFNs to posi-

tively influence host susceptibility to viral infec-
tions was tested in transgenic mice and cell lines.
Transgenic organisms overexpressing IFN-β gene con-
structs were shown to exhibit enhanced viral resis-
5   tance.

Recent progress in the understanding of signal
transduction pathways and transcription factors acti-
vated by IFNs and a variety of other cytokines promises
to open up new therapeutic approaches as well as novel
10   strategies of gene transfer treatments aiming at the
improvement of the immune response; i.e. the transfer
of cytokine encoding genes per se of distinct
"cytokine-specific" signaling components. Constitutive
expression of an interferon-stimulated gene factor
15   (ISGF2) also termed interferon regulatory factor (IRF-
1) transgenes has been reported to result in IFN-inde-
pendent activation of various IFN-inducible genes and
enhanced resistance to viral infection.

20   **Specific Disease Resistance Genes**

Other improvement which can be brought to ani-
mals by local gene transfer is specific disease resis-
tance. A well examined specific disease resistance gene
is the Mxl gene product of certain mouse strains. The
25   mouse Mxl protein belongs to a family of polypeptides
with GTPase activity synthesized in IFN-treated verte-
brate cells. Some Mx proteins have been shown to block
the multiplication of certain negative-stranded RNA
viruses, as for example Influenza virus , VSV, rhado
30   virus and Thogoto virus. Synthesis of mouse Mxl pro-
tein in various cell lines and transgenic mice demon-
strated that it is both necessary and sufficient to
promote resistance to influenza A viruses in previously
susceptible cells and animals. The cloning and func-
35   tional characterization of this specific disease resis-
tance gene enabled a gene transfer program to study

whether Mx1 transgenic pigs would show reduced suscep-
tibility to influenza infections.

Natural resistanc of certain inbred mouse
strains to infection with antigenetically unrelated
microorganisms such as *Mycobacteria, Salmonellae* and
Leishmania is controlled by a dominant locus on chromo-
some 1 called *Bcg, Lsh* or *Ity* respectively. The locus
affects the capacity of the host to restrict proliferan-
tion of these infectious pathogens during the non-spe-
cific macrophage-dependent phase of infection. A posi-
tional cloning approach resulted in the isolation of a
candidate *Bcg* gene designated *Nramp*. The reduction of
susceptibility to *Salmonella* infections by transgenesis
or gene therapy (*in vivo* or *ex vivo*) means is of great
value for animal production, especially poultry. Large
difference in resistance to *Salmonella* in chicken
inbred lines have been observed. Furthermore, natural
resistance or susceptibility to infection with
Mycobacteria in humans and *Brucella* in cattle has been
shown to be under genetic control similar to that
observed in inbred mice and governed by *Bcg*. Chronic
infection of cattle with *Brucella abortus* causes the
spontaneous abortion of fetal calves, threatening the
economic well-being of the dairy and beef industries.

Genetic resistance to certain retroviruses has
been observed as a polymorphic trait in several experi-
mental species. One of the identified loci in mice,
Fv-4, resembles the 3' half of a murine leukemia virus
extending from the end of the *pol* gene through a com-
plete *env* gene. Expression of *Fv*-4 encoding only the
viral envelope protein in transgenic mice conferred
resistance to infection with ecotropic retroviruses.
The mechanism of *Fv*-4 resistance is thought to be
related to the phenomenon of viral interference, i.e.
competition of the synthesized envelope protein with

exogenous virus for the virus receptor. Similar mecha-
nisms are used in antiviral strategies known as
"intracellular immunization".

      Expression of a transgene encoding an immu-
5 noglobulin specific for a common pathogen can provide
immunity for that pathogen. As shown by many investi-
gations, cloned genes coding for monoclonal antibodies
can be expressed in large amounts in genetically
manipulated mice. These mice produce antibodies
10 against specific antigens without prior contact or
immunization.

Intracellular Immunization

      The concept of "intracellular immunization"
15 essentially involves overexpression in the host of an
aberrant form (dominant-negative mutant) of a viral
protein that is able to interfere strongly with the
replication of the wild type virus. Elegant studies in
cultured cells resulting in acquired resistance to
20 various viruses include strategies preventing virus
attachment to the target cells, blocking the formation
of virus-host transcription complexes, expressing domi-
nant-negative viral trans-activators or interfering
with the assembly of infectious viral particles.

25   Endogenous mouse mammary tumor virus (MMTV)
proviruses have been found to co-segregate genetically
with loci termed self-superantigens identical to a pro-
tein encoded in the long terminal repeat of MMTV.
Genetically manipulated mice expressing high levels of
30 this self-superantigen were shown to be protected from
viral infection by deletion of a specific class of T-
cells which is the target for infection.

      The definition of "intracellular immunization"
is also applied for antiviral strategies described in
35 different connections such as expression of specific

resistance genes, antisense RNAs or other antiviral
components.

Recently, an "intracellular immunization"
approach carried out in farm animals was reported.
5   Transgenic sheep were produced and were shown express-
ing the visna virus envelope (env) gene. The visna
virus belongs to a subfamily of ovine retroviruses that
cause encephalitis, pneumonia and arthritis in sheep.
The env glycoprotein is responsible for the binding of
10   this virus to host cells. The target cell for visna
virus replication in infected sheep is the macrophage.
The expression of env protein on the cell surface of
visna-infected cells induces immune responses to the
virus. Expression of a gene construct consisting of
15   the visna U3 enhancer region fused to the env gene in
transgenic sheep had no obvious deleterious effect.
Thus, the genetically manipulated sheep lines provide
an evidence for the potential of a retroviral env gly-
coprotein to prevent infection and/or to modulate dis-
20   ease in its natural host after virus challenge.

Antisense RNA

The use of antisense RNA to inhibit RNA func-
tion within cells or whole organisms has provided a
25   valuable molecular biological method. Antisense RNA
functions by binding in a highly specific manner to
complementary sequences, thereby blocking the ability
of the bound RNA to be processed and/or translated.
Antisense sequences are considered an attractive alter-
30   native to conventional drugs in the therapy of micro-
bial infections, cancer, autoimmune diseases and other
malfunctions. Gene transfer experiments with antisense
constructs have been carried out in mice and rabbits.
Genetically manipulated mice expressing antisense RNA
35   targeted to the retroviral packaging sequences of
Molony murine leukemia virus did not develop leukemia

following challenge with infectious viruses. Trans-genic rabbits expressing an antisense construct comple-mentary to adenovirus h5 RNA were produced. Primary cells from these rabbits were found to be 90-98% more

5 resistant to adenovirus infection than cells from con-trol animals.

The use of antisense RNAs as anti-parasitho-genic agents can be developed to result not only in RNA-RNA hybrids but catallytically cleave a phosphodi-

10 ester bound in the target RNA strand. Four structural motifs (hammerhead and hairpin first identified in plant RNA pathogens, the delta motif found in human hepatitis delta virus and a less well characterized motif from *Neuspora*) have thus far been described as

15 intermediates in these self-cleavage reactions. By flanking the hammerhead motif of this ribozyme family with antisense sequences, the cleavage of specific tar-get RNAs has been demonstrated. A large number of sub-strate molecules can be processed by the catalytic RNA

20 because the ribozyme *per se* is not consumed during the cleavage reaction. Bovine leukemia virus (BLV), a retrovirus , causes persistent lymphocytosis and B-lym-phocyte lymphoma in cattle and sheep. A hammerhead ribozyme flanked by antisense sequences directed

25 against regulatory proteins of BLV was shown to inhibit BLV expression in persistently infected cells. This demonstrates the possibility of generating localized (*in vivo* or *ex vivo*) or generalized (transgenic ani-mals) gene therapies that will be resistant to BLV-

30 induced diseases.

Somatic Gene Transfer Approaches

Somatic gene transfer into farm animals will become more significant. *Ex vivo* and more recently *in*

35 *vivo* gene therapy has been applied for several genetic diseases in human. Current therapies developed for

more than 10 gene human disorders, such as failing
gen s coding normally for the adenosine deaminase, LDL
receptor, glucocerebrosidase, blood clotting factor
VIII, phenylalanine hydroxydase, dystrophin and others.
5   The efficiency of the gene therapy approach has no more
to be proved.

Novel methods for gene transfer into somatic
cells promise to be highly efficient. These include
viral vectors for delivering gene constructs and non-
10 viral technologies, such as micro-bombarding or injec-
tion of DNA particles or solutions into tissues or
blood vessels. Although most efforts are directed pri-
marily towards the possibility of treating human dis-
eases, some applications of somatic gene transfer could
15 be of great value in veterinary medicine. It makes
direct "genetic immunization" and other methods of
immunomodulation possible. "Genetic immunization",
i.e. application of DNA constructs encoding immunogens,
has at least two powerful uses. One is to simplify the
20 procedure and to shorten the time required to produce
antibodies to particular proteins by eliminating the
steps for protein purification. it would be more rapid
again to introduce a gene encoding directly a neutral-
izing or bacteriocid antibody in the organism. The
25 second is the genetic vaccination of animals against
infections by producing foreign antisense encoded by
appropriate gene construct.

The somatic gene transfer approach can now be
applied also both to cure and prevent an infectious
30 diseases by releasing in the organ, or in the organism,
a protein which is lethal and absolutely specific for
the targeted microorganism and without any affinity or
effect for the animal.

Such proteins or peptides having a high and
35 specific antimicrobial activity are divided into two

families, one including the bacteriocins and the other
the lanthionines, also called lantibiotics. The appli-
cation of biotechnology to animal treatment, particu-
larly farm animals, is opening up new avenues of pre-
5   vention and control that will have important implica-
tions. The bacteriocins consist of enzymes and other
bactericidal proteins. They act as catalysts and are
very specific to a single chemical reaction. Bacte-
riocins kill targeted organisms rapidly by lysing the
10 cell wall, and they do not require that the organism
undergo cell division. They are produced naturally by
bacteria as a means of population control. These pro-
teins are larger molecules than antibiotics and are
expected to persist in the treated organ longer. One
15 of these well known bacteriocins is lysostaphin, which
is   produced   by   *Staphylococcus   simulans*   biovar
staphylolyticus. Unlike antibiotics, the rapid action
of bacteriocins reduces the likelihood of an induced
resistance in target and non-target organisms. For
20 example, current research conducted so far seems to
indicate that bacteriocins used for mastitis treatment
are non-toxic to other organisms.

Lantibiotics   are   peptide-derived   antibiotics
with high antimicrobial activity against several patho-
25 genic bacteria. The ribosomal origin of lantibiotics
was first shown by the isolation of the structural
gene, *epiA*, for epidermin, a lantibiotic produced by
Staphylococcus epidermidis. The general structure of
lantibiotic genes is the same in all lantibiotics
30 described so far. The primary transcript of linear
lantibiotics is a prepeptide which consists of an
N-terminal leader sequence that is followed by the
C-terminal propeptide from which the lantibiotic is
matured and a characteristic proteolytic processing
35 site with proline at position -2. Nisin, produced by

several Lactococcus lactis strains, is a prominent mem-
ber of the group of lanthionines.

Other bacteriocins and lanthionines are ambi-
cins, defensins, cecropins, thionins, mellitins,
5   magainins, attacines, diphterins, saponins, cacrutins,
xenopins, subtilins, epidermins, pep5, lacticin 481,
ancovenins, duramycins, gallidermins, cinnamycins,
andropins and mastoparans.

Another new class of molecule complexes which
10   can be secreted by the transgene, i.e. the genetic con-
struct used for a gene therapy application is the immu-
noadhesins. The therapeutic potential of antibodies
has long been recognized Human antibodies should be
minimally immunogenic to the patient; they should
15   therefore be safe for chronic or repeated use. How-
ever, it can be difficult to generate useful human
antibodies for several reasons: it is ethically impos-
sible to immunize human beings for experimental pur-
poses, thus the available human antibodies are limited
20   to the products of inadvertent immunization or vaccina-
tion. Furthermore, there have been technical difficul-
ties in the immortalization of human cell lines. Per-
haps the most refractory technical problem is that many
applications require antibodies to human antigens;
25   since human antibodies with the desired specificity.

Several potential approaches exist to circum-
venting these problems. One approach is to engineer
the desired specificity of binding into human antibody
variable(V) regions. This can be done by deriving the
30   complementary determining regions either from mouse
antibodies, or from in vitro recombination combined
with selection (e.g. combinatorial libraries and phage
display technology). An alternative approach, which
sometimes has advantages, is to create an antibody-like
35   molecule by combining a binding site, derived from a

human protein such as a cell-surface receptor or cell-adhesion molecule, with antibody constant domains. Such molecules are known as immunoadhesins.

Immunoadhesins can possess many of the desired chemical and biological properties of antibodies. Examples exist of immunoadhesins that can bind to Fc receptors, mediate antibody-dependent cellular cytotoxicity, and show active transport across the primate placenta. Since the immunoadhesin is constructed from a receptor sequence linked to an appropriate hinge and Fc sequence, the binding specificity of interest can be achieved using entirely human components. Another potential foreign sequence is that in the joining region.

One of well studied immunoadhesins is CD4-IgG which as been found entirely non-immunogenic in human clinical trials. A second candidate for clinical use is a rumor necrosis factor receptor immunoadhesin (THFR-IgG); this molecule is particularly interesting, since the soluble receptor itself is found naturally in the body and has been considered as a possible therapeutic. While soluble receptors are valid clinical candidates, the IgG fusion form may well confer advantages such as longer half-life and improved avidity and affinity. Some receptors or immunoinducers that have been joined to the Fc part of IgG to form immunoadhesins are reported in the literature: T cell receptor, CD4, 1-selectin, CD44, CD28, B7, CTLA-4, CD22;, TNF receptor, NP receptor, IgE receptor, INF-γ receptor. These immunoadhesins should be useful in antigen recognition, reception to HIV, lymphocyte adhesion, receptor for hyluronidase, interaction B and T lymphocytes, inflammation, septic shock, homeostasis and allergy.

In animals, the advent of molecular biology techniques allow to create an immunoadhesin which could

has two specific activities. For example, in the goal
to eliminate a contamination with *Staphylococcus aureus*
it can be possible to have an immunoadhesin composed of
a lytic enzyme, like the lysostaphin, linked to the Fc
5   part of the human IgG which has a high affinity for the
protein A at the surface of the bacteria. Once the Fc
is linked to the protein A on *Staphylococcus aureus*,
the lytic part, the lysostaphin, can lyse the bacteria.

The gene therapy treatments can be applied in
10   such a way that the gene included in the constructs
transferred could be coding for an immunomodulator,
such as interleukins, chemokines, interferons, leu-
kotriens, and certain growth factors. As explained
before, the immunomodulators can makes the animal more
15   resistant to several microorganisms.

SUMMARY OF THE INVENTION

The present invention relates to the animal
gene therapy. Animal gene therapy means an approach by
20   which a DNA construct involving an inducible or consti-
tutive promoter linked to a gene coding for a curative
or protective protein or antisense RNA or peptide which
acts against infectious or potentially infectious
microorganisms responsible of the diseases. Disclosed
25   is a method for expressing a protein or antisense RNA
or peptide which directly or indirectly has a therapeu-
tic or prophylactic effects against infectious microor-
ganisms in an animals. The invention is useful for
producing a heterologous or homologous protein or
30   antisense RNA or peptide which is tethered to a spe-
cific tissue or organ and which can act on a microor-
ganisms infecting the animal. The method involves
inducing a liquid complex including a genetic construct
into a determined tissue of the animal. If desired,
35   the infused genetic construct can be treated with a

polycationic compound and/or a lipid to improve the
efficiency with which it is taken up by secretory cells
of the animals.

5   The most costly infectious disease in animals
is mastitis caused by the infection of the mammary
gland.   Among others, this invention relates to a
method of treating mastitis.   More particularly, this
invention relates to the use of DNA constructs designed
to be transcribed in a therapeutic protein after inser-
10   tion into the mammary gland of both lactating or non-
lactating animals.

Bovine, caprine, ovine and porcine mastitis
remain some of the most costly diseases in animal agri-
culture.   Mastitis represents a significant economic
15   loss to the diary industry, approximately 70 to 80 per-
cent of which can be attributed to a decrease in milk
production.   Many infective agents have been implicated
as causes of mastitis and these are dealt with sepa-
rately as specific entities in cows, sheep, goats and
20   pigs.

Despite significant progress in mastitis con-
trol due to widespread adoption of post-milking teat
antiseptisis, many herds continue to be plagued by this
disease.   A variety of different procedures have been
25   described and used to cure mastitis caused by bacteria
and yeast.   These procedures include the systemic immu-
nization of the infected animals with whole or partial
protein extracts of the infective agents in order to
stimulate the immune response of the treated animal to
30   these agents.   Antibodies generally produced in this
way act against a membrane protein, a binding protein
or a toxin secreted by the microorganisms.   Hence these
antibodies act as anti-adhesive, anti-toxin, neutraliz-
ing or opsonic molecules (Nordhaug et al., 1994, *J
35   Dairy Sci.*, **77**:1267 & 1276).   Nevertheless, the blood-

milk barrier prevents all but a very small proportion of circulation IgG antibodies from reaching mammary secretion during lactation.

Other procedures have been carried out in order
5 to stimulate the diapedesis and phagocytosis of con-taminating agents by leukocytes, more particularly polymorphonuclear neutrophils and macrophages. The stimulating molecules, which have been administered by intramammary injection, are cytokines, interleukin-1β,
10 interleukin 2, interferon-γ, tumor necrosis factor-α.

The most widely used procedure to cure infec-tious diseases is administration of antibiotics. How-ever, this approach inflicts a lot of side effects to the animal and particularly in the case of dairy ani-
15 mals, the milk must be discarded during the treatment period. Unfortunately, all current procedures are very short-lasting and consequently relatively inefficient. For example, none of the gram positive bacteria are entirely eliminated from the udder after treatments
20 with antibiotics.

For these reasons a gene therapy procedure is desired that allows a gene to be integrated into a tar-geted tissue, such as mammary gland, and provides for the elimination, by genetic therapy, of the contaminat-
25 ing microorganisms. In addition, gene therapy of the mastitic gland eliminates all the side effects of other procedures, enabling also an inserted gene to synthe-size inductively or constitutively in a permanent man-ner an effective amount of its therapeutic protein,
30 peptide or RNA antisense product. Therefore, this invention allows a much more specific and effective system of infectious diseases treatment than is cur-rently possible.

Additional objects, features, and advantages of
35 the invention will become apparent to those skilled in

the art upon consideration of the following detailed
description of preferred embodiments exemplifying the
best mode of the invention as presently perceived.

5   The present invention provides a recombinant
DNA which comprises a nucleotide sequence which encodes
a protein or polypeptide which is useful in the prophy-
laxis or treatment of mastitis, and at least one regu-
latory control element which allows for expression of
said nucleotide sequence in a mammary gland.

10   Suitable regulatory control elements include
transcription and translation regulatory sequences.
Transcription and translation regulatory sequences are
those DNA sequences necessary for efficient expression
of the product. In general, such regulatory elements

15 can be operably linked to any nucleotide sequence to
control the expression of the sequence, the entire unit
being referred to as the "expression cassette". Hence
the invention further provides an expression cassette
containing the above-mentioned recombinant DNA.

20   An expression cassette will typically contain,
in addition to the coding nucleotide sequence, a pro-
moter region, a translation initiation site and a
translation termination sequence.

         Unique endonuclease restriction sites may also
25 be included at the end of an expression cassette to
allow the cassette to be easily inserted or removed
when creating DNA constructs for use in transformations
as is known in the art.

         In particular the invention provides a DNA con-
30 struct designed to express a protein or polypeptide
which is useful in the prophylaxis or treatment of
infectious diseases after insertion into the targetted
tissues. Suitably the DNA construct comprises an
inducible or constitutive promoter which is linked to a
35 coding   nucleotide   sequence   or   gene   and   thereby

expresses a therapeutic or protective protein which acts against infectious or potentially infectious microorganisms responsible for the diseases of animals.

For example, such DNA constructs can be admin-
5   istered to both lactating or non-lactating animals for the prophylaxis or treatment or mastitis. Hence the invention further provides a method for the prophylaxis or treatment of mastitis which comprises transforma-tion of mammary gland tissue with a DNA construct as
10   described above.

The present applicants have found that expres-sion of proteins in mammary glands over an extended time period is possible and that a gene therapy approach to the problem of mastitis is feasible. Inte-
15   gration of a gene which encodes a therapeutic protein or polypeptide into mammary gland tissue would allow, for example, for the elimination of infective microor-ganisms by genetic therapy. In addition, gene therapy of the mastitis gland eliminates all side effects of
20   other procedures, also enabling an inserted gene to synthesize permanently and inductively or constitu-tively an effective amount of its therapeutic protein product. A gene therapy approach would be a much more specific and effective system of mastitis treatment
25   than is currently available.

Transformation of mammary gland tissue gener-ally requires that the DNA be physically placed within the host gland. Current transformation procedures use a variety of techniques to introduce naked DNA into a
30   cell and these can be used to transform a mammary gland. For example, the DNA can be injected directly into glands through the use of syringe. Alternatively, high velocity ballistics can be used to propel small DNA associated particles into the gland through an
35   udder's skin incision.

The DNA can also be introduced into a mammary
gland by insertion of other entities which contain DNA.
These entities include minicells, cells (e.g. fibro-
blasts, adipocytes, epithelial cells, myoepithelial
5   cells, mammary carcinoma cells, kidney cells),
liposomes (e.g. natural or synthetic lipid vehicles,
cationic liposomes) or other fusible lipid-surfaced
bodies. The entities are transformed *in vitro* prior to
insertion using the above-described DNA constructs.

10   Thus the invention also provides a cell which
has been transformed using a DNA construct as described
above. Examples of such cells include Mac-T cells.
Genetically transformed cells of this type are suitable
for reimplantation into a mammary gland to produce the
15   desired proteins or polypeptides.

Furthermore the invention provides a liposome
which incorporates the above-described DNA construct.

Introduction of the naked or complexed DNA con-
structs into the mammary gland can be performed by
20   direct injection through a skin incision of the udder
or through the teat canal.

Where appropriate, the DNA construct is admin-
istered in the form of a pharmaceutically or veterinary
acceptable composition in combination with a suitable
25   carrier or diluent. Suitable carriers are liquid car-
riers such as water, salts buffered saline or any other
physiological solutions. These compositions form a
further aspect of the invention.

The protein or polypeptides produced should be
30   effective prophylaxis or treatment of mastitis. Such
proteins or polypeptides include mucolytic proteins
such as enzymes, antibiotics, antibodies, cytokines,
tumor necrosis factors as well as proteins which can
induce an immune response to infective or potentially

infective agents and those which activate polymorphonu-
clear neutrophils, or macrophages.

In a preferred embodiment, the invention pro-
vides a recombinant DNA sequence which comprises a
5   nucleotide sequence which encodes a lytic protein or
antibody under the control of a mammary gland specific
promoter, or any ubiquitous or inducible non mammary
promoter.

The invention is particularly applicable for
10   the treatment of farm animals: bovine, caprine, ovine,
and porcine, but can concern also lower mammals or
lower milk producers: rabbit, camel and bison.   The
invention can also be used in humans to eliminate par-
ticularly most *Staphylococci*.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates examples of DNA constructs
in accordance with the present invention; and

Fig. 2 illustrates the rate of synthesis of
20   human growth hormone in milk's sheep after injection of
cationic liposome-DNA complex into the mammary gland.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with one preferred embodiment of
25   the present invention, animal gene therapy of infec-
tious diseases consists in transfecting a targeted tis-
sue with DNA sequences designed to produce molecules
which will be relargued into the organ or the organism,
this would than protect the animal against the infect-
30   ing or potentially infecting microbial agents.

In accordance with another embodiment of the
present invention, mastitis gene therapy of mammals
consists of transfecting the mammary glands with DNA
sequences designed to produce molecules which will be
35   relargued into the udder, this would than protect the

animal against the infecting or potentially infecting microbial agents.

The targeted tissue can also be transformed with other DNA sequences such as gene transcription and translation regulatory sequences. Transcription and translation regulatory sequences are those DNA sequences necessary for efficient expression of the gene product. In general such regulatory elements can be operably linked to any gene to control the gene's expression, the entire unit being referred to as the "expression cassette". An expression cassette will typically contain, in addition to the coding sequence, a promoter region, a translation initiation site and a translation termination sequence. Unique endonuclease restriction sites may also be included at the ends of an expression cassette to allow the cassette to be easily inserted or removed when creation DNA constructs.

The expression of a gene is primarily directed by its own promoter, although other DNA regulatory elements are necessary for efficient expression of a gene product. Promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs (bp) upstream of the transcription start site. In most instances the TATA box is required for accurate transcription initiation. By convention, the transcription start site is designated +1. Sequences expending in the 5' (upstream) direction are given negative numbers and sequences extending in the 3' (downstream) direction are given positive numbers.

Promoters can be either constitutive or inducible. A constitutive promoter controls transcription of a gene at a constant rate during the life of a cell, whereas an inducible promoter's activity fluctuates as determined by the presence (or absence) of a specific inducer. The regulatory elements of an inducible pro-

moter are usually located further upstream of the tran-
scription start site than the TATA box. Ideally, for
experimental purposes, an inducible promoter should
possess each of the following properties: a low to non-
5   existent basal level of expression in the absence of
inducer, a high level of expression in the presence of
inducer, and an induction scheme that does not other-
wise alter the physiology of the cells. The basal
transcription activity of all promoters can be
10   increased by the presence of "enhancer" sequences.
Although the mechanism is unclear, certain defined
enhancer regulatory sequences are known, to those
familiar with the art, to increase a promoter's tran-
scription rate when the sequence is brought in proxim-
15   ity to the promoter.

      Constitutive promoters can activate the tran-
scription of its linked gene in a tissue specific man-
ner, such as those naturally actives in the epithelial
cells of a mammary gland. For example, strong consti-
20   tutive promoters are those controlling the expression
of caseins, lactoglobulins, lactoferrin, lactalbumin,
lysosymes, whey acidic proteins (WAP) coding genes in
mammary glands. Preferentially, the promoters origi-
nates from domestic animals, bovine, caprine, ovine or
25   porcine species. Alternatively, specific mammary gland
promoters can originates from smaller animals, lagomor-
phes, rodents, felines or canines. Other constitutive
promoters regulating expression of the cytoplasmic β-
actin or ubiquitin genes can be used.

30   Viral or retroviral promoters can be used also,
like Cytomegalovirus (CMV), Simian virus 40 (SV40) or
mouse mammary tumor virus (MMTV, which is additionally
inducible).

      Inducible promoters include any promoter capa-
35   ble of increasing the amount of gene product produced,

by a given gene, in response to exposure to an inducer.
Inducible promoters are known to those familiar with
the art and a variety exist that could conceivably be
used to drive expression of the protective or curative
5   molecule's gene.

Two preferred inducible promoters are the heat
shock promoter (HST) and the glucocorticoid system.
Promoters regulated by heat shock, such as the promoter
normally associated with the gene encoding the 70 kDa
10   heat shock protein, can increase expression several-
fold after exposure to elevated temperatures. The heat
shock promoter could be used as an environmentally
inducible promoter for controlling transcription of the
protective or curative molecule's gene. The glucocor-
15   ticoid system also functions well in triggering the
expression of genes including protective or curative
molecule's gene. The system consists of a gene encod-
ing glucocorticoid receptor protein (GR) which in the
presence of a steroid hormone forms a complex with the
20   hormones. This complex then binds to a short nucleo-
tide sequence (26 bp) named the glucocorticoid response
element (GRE), and this binding activates the expres-
sion of linked genes. The glucocorticoid system can be
included in the DNA transformation construct as a means
25   to induce protective or curative molecule's expression.
Once the constructs have been inserted the systemic
steroid hormone or glucocorticoid will associate with
the constitutively produced GR protein to bind to the
GRE elements, thus stimulating expression of the pro-
30   tective or curative molecule's genes (e.g. antibodies
or enzymes).

Presumably the targeted tissue will allow the
inserted gene (naked, liposome, cell-enclosed or coated
solid particle) to produce its protein product in an
35   amount sufficient to produce the desired effect. The

inserted gene's products must cure or protect the organ
or the organism in which it is expressed against infec-
tious or potentially infectious microorganisms respon-
sible or potentially responsible of the disease.

5   The transformation of an animal tissue requires
that the DNA be physically placed within the host ani-
mal. Current transformation procedures utilize a vari-
ety of techniques to introduce naked DNA into a cell,
that can be used to transformed a targeted tissue. In
10   one form of transformation, the DNA is injected
directly into the tissue though the use of syringe.
Alternatively, high velocity ballistics can be used to
propel small DNA associated particles into the tissue
through a skin's incision. In other forms, the DNA can
15   also be introduced into a targeted tissue by insertion
of other entities which contain DNA. These entities
include minicells, cells (e.g. fibroblasts, adipocytes,
Mac-T cells, myoepithelial cells, mammary carcinoma
cells, kidney cells, liver cells, lung cells, lympho-
20   cytes, leukocytes), liposomes (e.g. natural or syn-
thetic lipid vehicles, cationic liposomes) or other
fusible lipid-surfaced bodies.

          The invention is concerned when a neutralizing,
lytic or opsonic molecules are synthesized from the
25   gene used for the infectious disease's gene therapy.
Preferentially, in the case of the mastitis, the gene
coding for a mucolytic protein (e.g. bacteriocins and
lanthionins) can be used to eliminates the Gram posi-
tive bacteria (mostly *cocci*).   The gene products can
30   serve as an immunomodulator and to induce an immu-
nologic response, the activation of polymorphonuclear
neutrophils, or macrophages for example.   The product
can be a cytosin or other immunomodulator.   Alterna-
tively, the genes can be used for in-situ synthesis of
35   the following therapeutic polypeptides:

1. Enzymes or mucolytic proteins, such as lysostaphin and mucolysins;

2. Antibodies, such as anti-hemolysins, anti-leucocidin, anti-protein A, anti-collagen, anti-fibronectin binding protein, anti-laminim, anti-α-toxin and anti-β-toxin antibodies; opsonic antibodies and antibodies raised against cell fusion viral protein;

3. Cytokines, interleukines, chemokines, growth factors;

4. Interferons;

5. Tumor necrosis factors; and

6. Immunoadhesins or immunotoxins.

While antibiotics are not very suitable, it can be alternatively used with inducible promoters.

Microorganisms which can be responsible of the mastitis and be eliminated by the gene therapy approach are:

In cattle *Streptococcus agalactiae, Str. ube, Str. zooepidemicus, Str. dysgalactiae, Str. faecalis* and *Str. pneumoniae, Straphylococcus aureus, Escherichia coli, Klebsiella spp., Corynebacterium pyogenes, Cor. bovis, Mycobacterium tuberculosis, Mycobacterium spp., Bacillus cereus, Pasteurella multocida, Pseudomonas pyocyaneus, Sphaerophorus necrophorus, Serratia marcescens, Mycoplasma spp., Nocardia spp.,* a fungus *Trichosoporon spp.,* yeasts *Candida sp., Cryptococcus neoformans, Saccharomyces,* and *Torulopsis spp.*.

In sheep: *Pasteurella haemolytica, Staph. Aureus, ActinoBacillus lignieresi, E. coli, Str. uberis* and *Str. agalactiae,* and *Cor. pseudotuberculosis.*

In goats: *Str. agalactiae, Str. dysgalactiae, Str. pyogenes*, and *Staph. aureus*.

In pigs: *Aerobacter aerogenes, E. coli, Klebsiella spp., Pseudomonas aeruginosa*, coagulase-positive *Staphylococci, Str. agalactiae, Str. dysgalactiae*, and *Str. uberis*.

In horses: *Corynebacterium pseudotuberculosis, Str. zooepidemicus*, and *Str. equi*.

Other microorganisms and diseases which can be eliminated from exotic animals by the method of gene therapy are those causing:

In primate: Poliomylltis, Measles, Mumps, Rubella, DPT, Tetanus.

In canidae: Can. distemper, Can. adenovirus, Can. parvovirus, Can. parainfluenza, Rabies, Leptospire bacterin.

In felidae: Fel. panleukopenia, Fel. rhinotracheitis, Fel. caliciviruses, Rabies.

In Artiodactyla: BVD, 8-way Clos. bacterin, 5-way Lepto. bacterin, Parainfluenza 3, Prions, Scatters.

Examples of infectious diseases which could be cured or prevented by the application of gene therapy are: anemia, arthritis, rhinotracheitis, bronchitis, bulbar paralysis, bursal diseases, hepatitis, cloacitis, coryza, enterohepatitis, hemopoietic necrosis, jaundice, keratoconjunctivitis, laryngotracheitis, myxomatosis, necrotic hepatitis, ophthalmia, pancreatic necrosis, pododernatitis, polyarthritis, pustular balanoposthitis, vulvovaginitis, serositis, sinusitis, stomatitis, synovitis, thromboembolic meningitis, and tracheobronchitis.

The present invention concerns a gene therapy approach with both curative and prophylactic activities on causing diseases infectious microorganisms. The

invention concerns in particular DNA sequences, expres-
sion vectors, DNA carriers (lyposome, solid particles)
and cells allowing to make use of the process.

5   The invention concerns equally the cells (e.g.
Mac-T, lung, kidney, muscle cells) genetically trans-
formed *in vitro* with the gene of interest and reim-
planted into the originating tissues to produce the
curative or prophylactic proteins, peptide or antisense
RNA against microorganisms responsible or potentially
10   responsible of the diseases.

The invention concerns more particularly domes-
tic animals: bovine, caprine, ovine, porcine, feline,
canine and birds, but can concerns also more exotic
animals such as rabbit, camel and bison.

15   The present invention will be more readily un-
derstood by referring to the following examples which
are given to illustrate the invention rather than to
limit its scope.

EXAMPLE I

20

Long-term persistence of plasmid DNA and foreign expression in sheep mammary glands

Mammary-gland promoters have been used in
transgenic animals to limit transgene expression to the
25   mammary gland. Gene therapy techniques to target just
one organ for introduction of a foreign gene have also
been demonstrated. Most efforts toward postnatal gene
therapy have relied on new genetic information into
tissues: target cells are removed from the body,
30   infected with viral vectors carrying the new genetic
information, and then reimplanted into the body. For
some applications, direct introduction of genes into
tissues *in vivo*, with or without the use of viral vec-
tors, would be useful. Direct *in vivo* gene transfer
35   into postnatal animals has been achieved with formula-
tions of DNA encapsulated in liposomes, DNA entrapped

in proteoliposomes containing viral envelope receptor
proteins (Nicolau et al., 1983, *PNAS USA*, **80**:1068),
calcium phosphate-coprecipitated DNA (B nenisty et
al., 1986, *PNAS USA*, **83**:9551), and DNA coupled to a

5 polylysine-glycoprotein carrier complex (Wu and Wu,
1988, *J. Biol. Chem.*, **263**:14621). *In vivo* infectivity
of cloned viral DNA sequences after direct intrahepatic
injection with or without formation of calcium phos-
phate coprecipitates has also been described (Seeger et

10 al., 2984, *PNAS USA*, **81**:5849). With the use of cat-
ionic lipid vesicles (Felgner et al., 1989, *PNAS USA*,
84:7413), mRNA sequences containing elements that
enhance stability can be efficiently translated in tis-
sue culture cells (Malone et al., 1989, *PNAS USA*,

15 **86**:6077) and in *Xenopus laevis* embryos (Malone, 1989,
Focus 11:61). It is demonstrated here that injection
of pure DNA complexed to cationic liposomes directly
into sheep mammary gland results in significant expres-
sion of reporter gene within the gland.

20 **Preparation of plasmid-liposome mixture**

Plasmid pCR3 (InVitrogen) was used as mammalian
expression vector. After PCR amplification, the human
growth hormone (hGH) cDNA was inserted into pCR3. This
resulted in plasmid construct pCR3. Plasmid-Lipofect-

25 AMINE™ (BRL) mixture was prepared as described by the
manufacturer (GibcoBRL). Briefly, 50 ug of pCR3-hGH
suspended in 500 µl sterile phosphate buffered saline
(PBS), was mixed to 100 µl of LipofectAMINE™ also pre-
viously diluted into 500 µl of PBS, and kept at room

30 temperature at 1 hour.

**Infusion of the plasmid-liposome complexes into sheep
mammary gland**

The circular pCR3-hGH plasmid-LipofectAMINE™

35 mixture was loaded into a glass syringe. Just after
dropping, by using a 20-gauge needle, the DNA-liposome

complex was infused directly through the udder's skin into the mammary parenchyma. One ml was injected into the right quarter of two ewes. The milk of the left glands was used as negative controls.

Analysis of sheep milk

Sheep were milked once daily by hand with the milk kept at -80°C until analyzed. The amount of hGH was measured by immunoassay (Immunocorp) after determining that the milk did not affect the accuracy of the assay. Aliquots (100 ul) of milk samples were analyzed.

RESULTS

hGH synthesized by injecting pCR3-hGH into the mammary gland was detected all along the lactating period, meaning about 60 days, as illustrated in Fig. 2. The concentration of hGH in the sheep's milk was relatively high during the first 5 days. At that time it was of 300 to 400 ng/ml (± 43 ng/ml). hGH concentrations in the milk from the left (control) gland was from 10 to 15 ng/ml for the two sheep everyday of the experiment. No important differences of concentration of hGH in milk samples were found between each ewes.

Conclusion

These results demonstrate that expression from plasmid DNA can persist in a sheep's mammary gland for at least 60 days. The unprecedented ability of plasmid DNA to stably express a foreign gene in a mammary gland throughout the lactating period of a sheep has important implications for gene therapy. The stable expression of circular plasmid DNA suggest that foreign acceleration or by viral transduction should also be stably maintained.

EXAMPLE II

Human growth hormone (hGH) secretion in goats' milk after direct transfer of the hGH gene into the mammary gland

An alternative route of introducing genes into the mammary parenchyma is through expansion of gene therapy techniques. In this study two Gibbon ape leukemia virus (GaLV) pseudotype retroviral vectors were used to transfer reporter genes into a goat's mammary secretory epithelial cells *in vitro* and *in vivo*.

Cells and tissue culture

MDBKs, a bovine kidney cell line and Mac-T cells, a bovine mammary epithelial cell line were used. Retroviral packaging cell lines used (φCre, PA317, and PG13/LNc8) were acquired from ATCC. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamycin (54 mg/ml) and 10% fetal calf serum, 37°C with 5% CO_2 /95% air.

Establishment of producer cell lines

A construct carrying the JR-gal neo- (Wang et al., 1991, *Cancer Res.*, 51:2642) was transfected into the ecotropic packaging cell line φCre by particle bombardment at 1 μg of DNA per mg of gold beads. Two days after bombardment, the supernatant was removed from these cells and centrifuged, and after the addition of Polybrene at 4 μg/ml, the retroviral solution was used to infect both amphotropic and GaLV pseudotype packaging cell lines. A plasmid carrying the retrovirus vector, MFG-hGH was cotransfected with pSV2neo at a ratio of 50:1 via particle bombardment into PA317s and PG13/LN c8s. Packaging cells producing retrovirus containing the hGH gene were selected by G418 resistance (400 μg/ml).

Virus producing cells

The PG13/LN c8 clones that yielded the highest levels of hGH produced from the target cell lines were chosen for the infusions into a goat's mammary glands. Each clone was passed three times into 200 100 mm-plates. Cellular supernatant was collected over a 3-day period, concentrated, and resuspended in DMDM with Gentamycin.

Induction of cell division and lactation of goats

Two 2-year-old (goats 1 and 2) and two 1-year-old (goats 3 and 4) virgin Saanen-crossbred goats were treated with exogenous steroids i.m. over a 14-day interval to induce mammogenesis and subsequent lactation.

Infusion of viral stocks into a goat's mammary glands

Polybrene was added to concentrated PG13/LN c8 MFG-hGH viral stock at 80 µg/ml and loaded into a syringe. By using a 22-gauge stub adapter, the retroviruses were infused up the right mammary teat on days 3, 5, 7, 9, 11, and 13 of the hormonal regimen for goats 1, 2, and 4 and goat 3 received infusions on days 3, 5, 7, 9, 10, and 13. The amount of viral solution was different for each animal, ranging from 8 to 20 ml, and was determined by the integral capacity of the gland. The left gland served as the intraanimal control and was infused with DMEM containing gentamycin. Retroviral stock used for the infusions was then assayed on several cell lines.

Analysis of goat's milk

Goats were milked twice daily by hand with the morning milk kept at -80°C until analyzed. The amount of hGH was measured by immunoassay after determining that the milk did not affect the accuracy of the assay.

Aliquots (5 µl) of milk samples diluted 1:10 in double
distilled water were also analyzed by SDS/PAGE on 14%
gels stained with Coomassie blue. The protein concen-
tration of the milk samples was determined by using BCA
5   (Pierce et al., 1977, *Anl. Biochem.*, **81**:478).

RESULTS

Vector production of packaging cell lines

          The concentration of hGH in the medium removed
10   from Mac-T and MDBK cells 2 days after infection with
retrovirus packaged by PG13/LN c8 clone 6 was 192 and
3.8 ng/ml, respectively.   Twenty-eight days after
infection, hGH levels from these cells were 119.3 and
4.5 ng/ml, indicating that the provirus LTR was still
15   functioning 4 weeks after infection.

**Infusion of viral stocks into the mammary glands of
goats**

          Viral stock infused on day 13 for goats 1 and 2
20   was found to contain hGH at 224 ng/ml, indication that
the PG13/LN c8 packaging cell were also producing hGH.

Analysis of goat milk

          Lactation commenced on day 14 of the hormonal
25   regimen, 24 hr after the last viral infusion.   Milk
appeared normal throughout the lactations. The volume
of milk obtained from each udder half was approximately
150 ml on the first day of lactation for goats 1 and 2
but only 10 ml for goat 3, and 35 ml for goat 4. Milk
30   volume produced by each gland for all four goats
increased daily. The levels of hGH were determined by
immunoassay with unique hGH secretion patterns for each
animal. In goat 1, concentration of hGH dropped stead-
ily until day 9 of lactation when it leveled at 3-5
35   ng/ml, whereas goats had a more precipitous decrease in
measured hGH from day 1 to day 2 of lactation, though

the animal's production of hGH stabilized at 2-3 ng/ml
around day 10. Milking was stopped on day 15 of lacta-
tion for goats 1 and 2. Levels of hGH in the milk of
goat 3 dropped dramatically from day 1 to 2 of lacta-
5   tion and then increased from day 8 to day 9 where it
remained at 23 ng/ml until day 16 when it began to fall
again. Goat 4, in which prostaglandin E2 was infused
at the end of the remaining 19-day lactation after a
decline on the first 2 days. In addition, goat 4 was
10   still secreting hGH at 5 ng/ml after 28 days. hGH con-
centrations in the milk from the left (control) gland
ranged from 0.0 to 0.6 ng/ml for the four goats at all
evaluated times. These numbers are at the detection
level of the assay and correlate with ones measured in
15   two other lactating goats that had no exposure to
retrovirus. The total production of hGH in the four
animals ranged from 0.3 to 2 ug/day.

If the hGH gene had been stably incorporated
into the stem-cell population, it would have been
20   expected that the goats would also secrete hGH in a
second lactation after the gland had undergone involu-
tion. A second lactation was induced in two of the
goats, and though goat 1 did not produce hGH, goat 2
began secreting detectable amounts of hGH starting on
25   day 5 from the right (infused) gland and during the
subsequent 10 days hGH concentrations varied from 0.4
to 2.3 ng/ml. Milk from the left control gland during
this lactation always had no detectable levels of hGH.

SDS/PAGE of goat's milk sampled throughout the
30   period of collection showed no consistent differences
in the protein profiles from the retroviral-infused
right glands, the control left glands, and a goat not
exposed to the retrovirus. Protein concentrations
measured by BCA of the milk with hGH were not statisti-
35   cally different from the control milk, thus production

of hGH by the mammary secretory epithelial cells did not appear to affect the normal cellular protein machinery. There was an indication that the milk's proteins in the treated gland were not secreted at
5   maximal concentration on day 1 of lactation.

Conclusion

Applying gene therapy technology and replication-defective retroviral vectors to directly introduce
10 a foreign gene into a ruminant mammary gland has dramatically reduced the time of production of pharmaceuticals in milk, from years to weeks. Although the levels of expression found are low, the methods might find application in the evaluation of different gene con-
15 structs as a prelude to production of transgenic animals or in the production of low levels of important proteins for evaluation purposes.

EXAMPLE III
20

Effect of lysostaphin on *Staphylococcus aureus* infections on the mouse's mammary gland

Lysostaphin is an endopeptidase produced by *Staphylococcus simulans*. It hydrolyzes the pentaglycine
25 links of the peptidoglycan of members of the genus *Staphylococcus* and consequently has little activity against other prokaryotes and none against eukaryotes. The lysostaphin gene has been cloned and expressed successfully in *Escherichia coli* and *Bacillus* species
30 (Heath et al., 1987, *FEMS Microbiology Letters*, **44**:129; Heinrich et al., 1987, *Molecular and General Genetics*, **209**:563; Recsei et al., 1987, *PNAS USA*, **84**:1127). The use of lysostaphin to promote lysis of *Staphylococcus aureus* in a variety of experimental situations is well
35 known but the progress made in cloning and expressing the gene in other hosts raises the possibilities of

producing large quantities of the enzyme relatively
inexpensively. This may permit its use *in vivo* in new
approaches to the control of staphylococcal mastitis,
an economically important disease of lactating rumi-

5   nants (Bramley et al., 1990, *Res. Vet. Sci.*, **49**:120).
This experiment shows the use of a mastitis model in
the lactating mouse and clearly demonstrates potent
antibacterial activity of lysostaphin against *S. aureus*
in vivo.

10   Lysostaphin (Sigma Chem.) was dissolved in
skimmed milk (Oxoid) to provide a range of concentra-
tions between 0.1 and 100 µg/ml. Controls without
lysostaphin were included. One ml volumes of the con-
trols and lysostaphin dilutions were inoculated with

15   10^8 colony forming units (cfu) of *S. aureus* M60. This
strain produces both α and β toxins and was isolated
from a case of bovine mastitis. Lysostaphin concentra-
tions exceeding 2 to 3 ug/ml in milk produced a 2 to 3
$\log_{10}$ reduction in viable *S. aureus*, whereas 10 ug/ml

20   in milk reduced *S. aureus* from a mean of 7.95 $\log_{10}$/ml
in the control to 2.0 $\log_{10}$/ml. Consequently a dose of
10 ug of lysostaphin was selected for use *in vivo*.
Anaesthetized mice, of strain MF1, were inoculated in
the upper pair of abdominal mammary glands (designated

25   R4 and L4). Eight lactating mice were inoculated with
10^8 cfu of *S. aureus* in 0.1 ml saline in both R4 and
L4. This was followed one hour later by the infusion
of 10 ug lysostaphin in 0.1 ml saline into R4 and 0.1
ml saline into L4. After a further 30 minutes the mice

30   were killed and the mammary glands were aseptically
removed and homogenized in saline containing 0.1 mg/ml
trypsin (Sigma Chem.) to destroy active lysostaphin.
Ten fold dilutions were placed on 7 per cent calf blood
agar (Oxoid Blood Agar Base Number 2), incubated at

35   37°C overnight and viable counts determined. In a fur-

ther experiment using 20 mice a prophylactic use of lysostaphin was simulated by infusing 10 ug of lysostaphin intramammarily, followed either immediately or after one hour by 10^3 cfu of *S. aureus*. Control

5   glands were infused with saline instead of lysostaphin. After 24 hours the mice were killed and dissected. Gross pathological changes were noted an viable *S. aureus* counts determined as described above.

10   **RESULTS**

Infusion with 10mg lysostaphin into mammary glands previously inoculated with *S. aureus* reduced bacterial recoveries, compared to the controls, by more than 99 per cent in 30 min. This reduction was statis-

15   tically significant (t=2.56; P<0.02). When 10 ug of lysostaphin was administered either immediately or one hour before *S. aureus* inoculation, recoveries after 24 hours averaged around 10^2 viable *S. aureus* per mammary gland compared with approximately 10^9 per mammary gland

20   for the saline treated controls. In the latter case, the control glands showed severe pathological changes typical of acute staphylococcal mastitis in the mouse. The control glands were darker and reddened, had a brittle texture and some areas of liquefaction and

25   haemolysis. Histological sections revealed a severe inflammation, infiltration of neutrophils and macrophages with areas of coagulative necrosis. Large numbers of *Staphylococci* were visible. In contrast, the lysostaphin treated glands remained pale and elastic

30   with only slight reddening around the base of the teat. Histological examination showed little or no cellular infiltration, a well preserved and functioning alveolar structure and few *cocci*.

Conclusion

These experiments clearly demonstrate the anti-staphylococcal activity of lysostaphin *in vivo*. Both a therapeutic and prophylactic potential were demon-
5 strated. The cloning of the lysostaphin gene may make it readily available for therapeutic use at a competitive price and its relatively high specificity makes it attractive for use in food-producing animals. Furthermore, advances in transgenic technology allow the
10 direction of the expression of transgenes to the mammary gland of ruminants (Simons et al., 1987, *Nature*, **328**:530). In general, this has been applied to the production of pharmacologically active substances for use in human medicine. However, the incorporation and
15 expression of the lysostaphin gene in the lactating mammary gland could potentially increase the resistance of the animal to staphylococcal mastitis.

EXAMPLE IV
20

Lysostaphin efficacy for treatment of *Staphylococcus aureus* intramammary infection

Cloned-derived lysostaphin was evaluated as to its bactericidal effect on *S. aureus* intramammary
25 infections. *S. aureus* (Newbould 305) was eliminated from glands of guinea pigs 48 hrs post-infection by 125 µg of lysostaphin in 14/16, 25 µg in 5/8, 5 µg in 5/10, 1 µg in 0/1, and 0 µg in 0/3. Glands infected with *S. aureus* at 48 hours post-challenge in untreated guinea
30 pigs persisted, however, 3/25 control glands of treated guinea pigs cleared in response to treatment of the adjacent gland.

Somatic cell/ml in guinea pig shifted from 10^4
pre-infected glands to cell counts greater than 3×10^6
following *S. aureus* inoculation. Treatment with
lysostaphin caused a neutrophilic shift in the treated
5   gland to levels exceeding 108 accompanied by an
increase in the adjacent non-treated gland but dropped
sharply to pre-treatment level. The greatest response
in control glands was observed in animals receiving 125
ug which corresponded to 2/25 clearance of *S. aureus* in
10 control glands.

The leukocyte response to intramammary treat-
ment in the cow is similar to the guinea pig model
described above. Somatic cell levels increased ten-
fold in *S. aureus* infected glands at the milking fol-
15 lowing treatment. Cell levels returned to pre-treat-
ment levels or lower in subsequent milking. A rise in
leukocytes alone could not account for clearance of the
infection.

20   **EXAMPLE V**

**Use of a recombinant bacterial enzyme (Lysostaphin) as
a mastitis therapeutic**

A recombinant mucolytic protein, lysostaphin,
25 was evaluated as a potential intramammary therapeutic
for *Staphylococcus aureus* mastitis in dairy cattle.
Lysostaphin, a product of *Staphylococcus simulans*,
enzymatically degrades the cell wall of Straphylococcus
aureus and is bactericidal.
30   Thirty Holstein-Friesian dairy cattle in their
first lactation were infected with *Staphylococcus
aureus* (Newbould 305, ATCC 29740) in all quarters.
Infections were established and monitored for somatic
cell counts and *Staphylococcus aureus* colony-forming
35 units 3 weeks prior to subsequent treatment. Infected
animals were injected through the teat canal with a
single dose of recombinant lysostaphin (rLYS) (dose 1

to 500 mg) or after three successive p.m. milking with 100 mg of rLYS in 60 ml of sterile phosphate-buffered saline. Animals were considered cured if the milk remained free of *Staphylococcus aureus* for a total of
5   28 milkings after the last treatment.

RESULTS

Kinetic analysis of immunologically active rLYS demonstrated that a minimum bactericidal concentration
10 was maintained in the milk for up to 72 hours at 37°C. In contrast, penicillin G retained less than 10% of its bacteriostatic activity over the same incubation time.

Dose titration and kinetics of rLYS in the bovine mam-
15 **mary gland**

In order to determine the optimal effective dose to elicit long-term cures, a titration was per- formed in which a single dose of rLYS at concentrations of 0, 1, 10, 100, or 500 mg was administered.
20 Untreated quarters and the 1-mg treatment failed to clear all quarters of *S. aureus*. The 10- 100- and 500- mg does transiently cleared the milk of *S. aureus* for at least one milking. In relapsed quarters, the length of time of the milk remained clear of *S. aureus* was
25 approximately proportional to the dose administered. Fourteen days after treatment, two quarters were cured with the 100 mg dose and one with the 500 mg dose. Because rLYS maintains a minimal bactericidal concentration (MBC) for approximately 24 h and the
30 experimental infections undergo a 2- to 4- days cycling, multiple infusions of 100 mg of rLYS over three consecutive milking were determined to be optimal to maintain a minimal effective dose for 3 to 5 days and to elicit cures.
35

Conclusion

Staphylococcus aureus is one of the primary etiologic agents of bovine mastitis and a major cause of economic loss to the dairy industry. An effective

5 mastitis therapy for the lactating dairy cow remains a major unfilled need. Because current therapy is only moderately efficacious and is costly because of milk discard and culling infected animals, treatment only during the dry period has been the adopted herd manage-

10 ment practice of choice. Neither approach addresses the majority of the infections in a lactating animal, which are chronic and subclinical in nature. A recombinant protein such as rLYS with bactericidal activity against *S. aureus* could be an extremely useful thera-

15 peutic to the veterinarian. If rLYS was as efficacious as antibiotics, natural proteolysis and inactivation in the milk of rLYS, as well as inactivation during ingestion by the consumer, would potentially minimize any concerns associated with residues in milk.

20 The *in vivo* does titration suggested that the minimal effective therapeutic dose was 100 mg of rLYS. However, therapeutically, it would be desirable to administer multiple infusions of rLYS to maintain a minimal bactericidal activity within the milk of

25 treated glands for one to three successive milkings. The *in vivo* bactericidal activity of rLYS was most effectively demonstrated by the fact that 95% of the quarters cleared the milk of detectable *S. aureus* for a minimum of one milking after the last intramammary

30 infusion.

EXAMPLE VI

Expression of jet-injected plasmid DNA in the ovine mammary gland

35 A jet-injection based DNA delivery system has been evaluated as a means to transiently transfect the

lactating mammary gland *in vivo* and as a technique for
DNA vaccination.   The model expression plasmid con-
tained the human growth hormone (hGH) gene driven by
the human cytomegalovirus immediate early gene 1 pro-
5   moter/enhancer region (CMV). Expression from the naked
plasmid DNA jet-injector into lactating mammary glands
of sheep was sufficient to be detected by Northern blot
analysis when tissue was obtained 48 hours after *in
vivo* transfection. In conclusion, the ability to tran-
10   siently transfect lactating mammary tissue *in vivo* cir-
cumvents the difficulties encountered with *in vivo* cul-
ture techniques and provides a method for examining
mammary regulatory elements and testing of fusion gene
constructs designed for the production of transgenic
15   animal bioreactors.

EXAMPLE VII

Elimination of *Staphylococcus aureus* in an eukaryotic
20   system expressing the lysostaphin

The lysostaphin gene was introduced into 293
cells (human fetal kidney cells) maintained *in vitro*.
The recombinant bacteriocin, the lysostaphin, was
secreted in the medium culture and was found to kill
25   contaminant *S. aureus* during the challenge.

The lysostaphin gene was obtained by PCR ampli-
fication from extracted DNA of *Staphylococcus simulans*
biovar staphylolyticus (NRRL B-2628), and
Staphylococcus aureus strain Newbould (ATCC) was used
30   for the challenge in transfected eukaryotic cells.
Staphylococcal strains were grown in Brain Heart
Infusion (BHI) medium.

Purification of the lysostaphin gene
35   *Staphylococcus simulans* biovar *staphylolyticus*
was cultured overnight in a stirring incubator at 37°C.
The media was centrifuged, and the pellet was resus-

pended in 5 ml of 50 mM EDTA-50mM Tris-HCL (pH 7.8)
containing 50 mg of lysostaphin (Sigma) ml^{-1} and the
suspension was incubated at 37°C for 2 hours. Purified
bacterial DNA was directly amplified by PCR method to

5   isolated the lysostaphin gene. The set of oligonucleo-
tide   primers   used   were   as   followed:
5'-TTAAGGTTGAAGAAAACAATT-3'   (SEQ   ID   NO:1)   and
5'-GCGCTCACTTTATAGTTCCCCAA-3'   (SEQ   ID   NO:2).   The
amplification   was   performed   by   using   a   Thermal   DNA

10   cycler   and   2.5   units   of   Taq   DNA   polymerase   (Perkin
Elmer Cetus), and a 30 cycles program with an annealing
step at 60°C for 30 sec., elongation at 72°C for 90
sec. and denaturation at 93°C for 10 sec. The PCR
product   was   composed   by   the   entire   lysostaphin

15   sequence,   including   the   coding   gene   with   the
aminoterminal   pre-   and   pro-   regions.   All   other
recombinant   DNA   procedures,   including   restriction
endonuclease digestion, ligation, washing with phenol-
chloroform mixture, ethanol precipitation, transforma-

20   tion and cloning of the constructs in _E. coli_ strain
DH5a,   were   carried   out   by   standard   methods.   All
enzymes were from Boehringer Mannheim.

        The lysostaphin was linked to an eukaryotic
expression vector including the human cytomegalovirus

25   immediate early gene 1 promoter/enhancer region (CMV)
and the human interleukin-2 signal peptide.

Cell culture and DNA transfection

        293 cells, a human foetal kidney cell line

30   transformed by an origin-defective mutant of simian
virus 40, were cultured in Dulbecco's modified Eagle
medium (Sigma) supplemented with 10% (vol/vol) fetal
calf serum (Gibco BRL) and glutamine (1.4 mM). The
cells were seeded into 30-mm wells at 500 000 cells par

35   well and grown in 2 ml of medium for 24h at 37°C (in
air atmosphere containing 5% CO_2) to yield 50 to 60%

introduced into the cells by the calcium phosphate
method with the following modifications. The precipi-
tate containing 7.5 µg of DNA was added to 2 ml of cul-
ture medium. After 24 h, the medium was replaced with
5   2 ml of medium per well, and samples of the medium were
harvested at each 24 h following transfection to evalu-
ate the production of the lysostaphin by Western blot
analysis and ELISA

10 **Assay for biological active lysostaphin**

The wells containing the transfected 293 cells
were infected with 10^2 or 10^3 of *Staphylococcus aureus*
Newbould. Samples of 100µl of the infected medium were
spreaded on sheep blood agar. After incubation for 24
15 h at 37°C, the number of colony forming units (CFU) was
evaluated to assess the inhibition effect of the recom-
binant lysostaphin on the growth of the bacteria.

RESULTS
20

**Production of recombinant lysostaphin by transfected
eukaryotic cells**

The modified lysostaphin gene was transfected
into tissue culture cells to demonstrate the expres-
25 sion, processing and activity of the enzyme on infect-
ing bacteria. After analysis of the culture medium, a
band of approximately 25 kDa was generated; this band
was similar in size to mature lysostaphin. The same
result was observed in other experiments in which the
30 expression of recombinant lysostaphin has been carried
out in eukaryotic cells. The ELISA assays have
revealed that the recombinant lysostaphin was produced
in concentrations of 100 to 250 ng/ml/24h depending of
the clone.
35

Activity of the lys staphin secreted by mammalian cells

The activity of the recombinant lysostaphin secreted by transfected mammalian cells has been observed by its efficiency to reduce or in some repli-

5   cates to inhibit the growth of infecting *Staphylococcus aureus* in the culture media. Samples of media taken from non-transfected cells have shown none inhibitory effect on the development of the bacteria present in the wells. The plates of agar were completely conflu-

10   ent after overnight incubation. In contrast, when an initial amount of 10^3 bacteria was cultured in presence of transfected eukaryotic cells, very few CFU were counted on the plates. Less than 100 CFU were observed in our assays when 10^3 bacteria were used, while we did

15   not observed the presence of CFU on gels when 10^2 bac-teria were added to the wells containing the trans-fected cells.

While the invention has been described in con-nection with specific embodiments thereof, it will be

20   understood that it is capable of further modifications and this application is intended to cover any varia-tions, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure

25   as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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        (F) POSTAL CODE (ZIP): G6Z 2S8

    (ii) TITLE OF INVENTION: ANIMAL GENE THERAPY

    (iii) NUMBER OF SEQUENCES: 2

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: GB 9509461.1
        (B) FILING DATE: 10-MAY-1995

(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTAAGGTTGA AGAAAACAAT T   21

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid

                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

              (ii) MOLECULE TYPE: cDNA

              (iii) HYPOTHETICAL: NO

              (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCGCTCACTT TATAGTTCCC CAA   23

WE CLAIM:

1.   A method of treatment and/or prevention of an infectious disease in an animal, which comprises the steps of:

    a) producing a recombinant DNA expression system comprising at least a DNA sequence encoding for a therapeutic protein, peptide or antisense RNA operably linked to a promoter capable of directing the *in vivo* expression of said DNA sequence of a therapeutically effective amount of said protein, peptide or antisense RNA; and

    b) introducing into the animal the DNA expression system of step a) for expression of said therapeutic protein, peptide or antisense RNA.

2.   The method of claim 1, wherein said treatment and/or prevention of the disease is effected *in situ* and said DNA expression system is introduced in targeted tissue.

3.   The method of claim 1, wherein said DNA expression system is transgenic recombinant animal cells.

4.   The method of claim 3, wherein said cells are selected from the group consisting of epithelial mammary gland cells, blood cells, lymphocytes, leukocytes, T-lymphocytes, B-lymphocytes, erythrocytes, muscle cells, hepatic cells, kidney cells, lung cells, secretory cells and non-secretory cells.

5.   The method of claim 3, wherein said DNA expression system is selected from the group consisting of a lipidic liposome, a cationic liposome, an anionic liposome.

6.   The method of claims 1, 2, 3, 4 or 5, wherein said DNA sequence and said promoter are inserted into an expression vector.

7.   The method of claim 6, wherein said vector is a viral vector or a retroviral vector.

8.   The method of claims 1, 2, 3, 4, 5, 6 or 7, wherein said infectious diseases are caused by bacteria, virus, retrovirus, parasite, fungi, mold, yeast, prions or scrapies.

9.   The method of claim 8, wherein said therapeutic protein, peptide or antisense RNA are selected from the group consisting of bacteriocins, lanthionins, lactoferrin, lysosyme.

10.   The method of claim 9, wherein said bacteriocins and/or lanthionins are ambicins, defensins, cecropins, thionins, mellitins, magainins, attacines, diphterins, saponins, cacrutins, xenopins, subtilins, epidermins, pep5, lacticin 481, ancovenins, duramycins, gallidermins or cinnamycins.

11.   The method of claim 8, wherein said therapeutic protein, peptide or antisense RNA is selected from the group consisting of immunoglobulins, lactoglobulins, α -lactalbumin, bile-salt-stimulated lipase or ribosyme, cytokines, chemokines, growth factors, immunomodulators and major histocompatibility complex (MHC) proteins.

12.   The method of claim 3, which further comprises a 5' and 3' expression regulation DNA sequence and a secretory DNA sequence functional in said animal cells

and operably linked to the recombinant DNA encoding said therapeutic protein, peptide or antisense RNA.

13.   A non-human transgenic animal for the production of a recombinant protein, peptide or antisense RNA systemically or in targeted tissue, which comprises at least an expression regulation DNA sequence and a secretory DNA sequence encoding a secretory signal sequence operatively linked to a DNA sequence encoding said protein, peptide or antisense RNA for systemic expression or for expression in targeted tissue cells of said animal.

14.   The non-human transgenic animal of claim 13, wherein said expression regulation DNA sequence is selected from the group consisting of a constitutive promoter, an inducible promoter, a cytomegalo virus promoter.

15.   The non-human transgenic animal of claim 14 wherein said promoter is selected from the group of DNA sequences encoding lactoferrin, serum albumin, αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin, whey acidic protein, β-lactoglobulin, cytokines, chemokines and growth factors.

16.   The non-human transgenic animal of claim 13, wherein said secretory signal sequence is selected from the group consisting of DNA sequences encoding lactoferrin, serum albumin, αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin, cytokines, chemokines or growth factors.

17.   The non-human transgenic animal of claim 13, wherein the expression regulation and secretory signal

sequenc s are from human, bovine, caprine, ovine,
feline, canine, lagomorphes, birds and fishes.

18.   The non-human transgenic animal of claim 13,
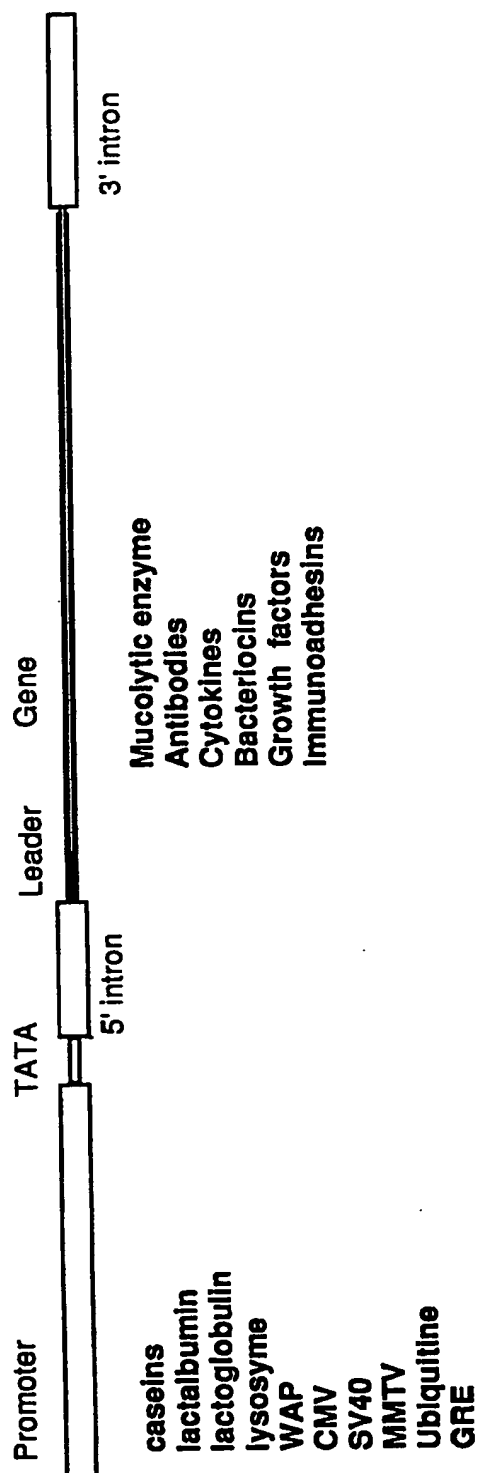wherein the promoter is tissue-specific for expression
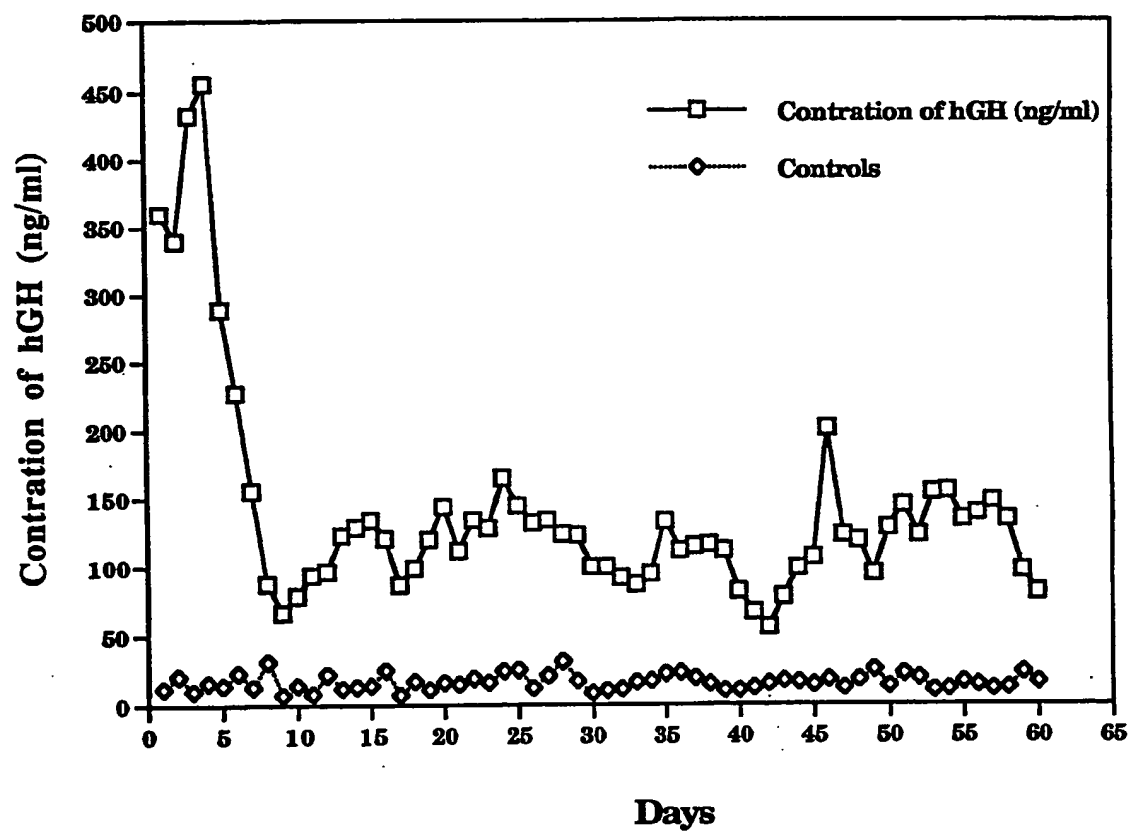in targeted tissue.

Fig. 1

2/2

Fig. 2

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/55   A61K48/00   A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   A61K   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	APPLIED AND ENVIROMENTAL MICROBIOLOGY, vol. 60, no. 3, March 1994, pages 771-776, XP000579799 WILLIAMSON ET AL: "EXPRESSION OF THE LYSOSTAPHIN GENE OF STAPHYLOCOCCUS SIMULANS IN A EUKARYOTIC SYSTEM"	1-9, 12-18
Y	see whole document,noting especially comments in last paragraph of page 775	10

X	WO,A,93 25567 (GENPHARM INT ;DEBOER HERMAN A (NL); STRIJKER REIN (NL); HEYNEKER H) 23 December 1993	1-9, 11-18
Y	see page 6, line 15 - page 9, line 16 see page 12, line 15 - page 31, line 13	10

	-/--	

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 September 1996	16. 09. 96.

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

Form PCT/ISA/210 (second sheet) (July 1992)

2

Inter   ional Application No

PCT/CA 96/00297

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 279 582 (BAYLOR COLLEGE MEDICINE) 24 August 1988	1-9, 11-18
Y	see column 3, line 31 - column 5, line 26 see column 6, line 45 - column 9, line 21 ---	10
X	TRENDS IN BIOTECHNOLOGY, vol. 11, no. 5, May 1993, pages 211-215, XP000389867 NABEL ET AL: "DIRECT GENE TRANSFER FOR IMMUNOTHERAPY AND IMMUNIZATION" see the whole document ---	1-8, 11-18
Y	WO,A,91 18512 (UNIV WASHINGTON) 12 December 1991 see page 9, paragraph 4 - page 10, paragraph 1 see page 11, paragraph 6 - page 12, paragraph 1 ---	10
Y	EP,A,0 448 511 (CIBA GEIGY AG) 25 September 1991 see page 2, line 42 - page 3, line 16 see page 6, line 11 - line 34 -----	10

2

INTERNATIONAL SEARCH REPORT

Box I   bservations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   1-12
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although these claims are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                              [ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9325567	23-12-93	AU-B-	4537393	04-01-94
		EP-A-	0652889	17-05-95
		JP-T-	8504562	21-05-96
EP-A-0279582	24-08-88	AU-B-	618524	02-01-92
		AU-B-	1178488	18-08-88
		AU-B-	661696	03-08-95
		AU-B-	1941092	24-09-92
		CA-A-	1321764	31-08-93
		JP-A-	63309192	16-12-88
		US-A-	5304489	19-04-94
WO-A-9118512	12-12-91	AU-B-	650459	23-06-94
		AU-B-	7953191	31-12-91
		CA-A-	2083595	26-11-91
		EP-A-	0532650	24-03-93
		US-A-	5378819	03-01-95
EP-A-0448511	25-09-91	AU-B-	655579	05-01-95
		AU-B-	7279791	19-09-91
		CA-A-	2037806	13-09-91
		JP-A-	4217903	07-08-92
		US-A-	5348743	20-09-94